

EFFECT OF FREEZE-DRIED BOVINE BONE XENOGRAFT ON TUMOR NECROSIS FACTOR- ALPHA SECRETION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Abstract– Alveolar bone augmentation requires the use of bone graft particles to promote bone formation. Freeze-dried bovine bone xenograft (FDBBX) is a type of bone substitute may be potential as an alternative to autogenous bone graft. However, since it is xenogeneic material, it may trigger body's immune response and cause early resorption of the graft. Tumor Necrosis Factor- α (TNF- α) is a cytokine which is released rapidly after trauma or infection and is one of the most abundant mediators in inflammation tissue. The immune system and immune response play a very important role in the concept of bone healing. Human peripheral blood mononuclear cells (hPBMCs) is a critical component of the immune system which release TNF- α . This study aims to evaluate FDBBX effect on the secretion of TNF- α in hPBMCs culture. hPBMC cultures were divided into two groups. In experimental groups, the cell was cultured in FDBX conditioned medium of 2.5% dilution while in control group, basic medium was used. After 1, 3, 5 days of culture, the cells were harvested for ELISA to analyze TNF- α secretion. TNF- α secretion in experimental groups was found significantly higher than that of control groups on 1, 3, and 5 days of observation. However, there were consistent decline in TNF- α secretion in both groups along observation periods. Freeze-dried bovine bone xenograft may induce increase of TNF- α secretion *in vitro* but does not cause prolonged inflammatory response.

INTRODUCTION

Alveolar bone grafting started to be more familiar between clinicians to overcome the problems that happened after tooth loss. It can be used in stimulate wound healing and stabilizing the dimensions of the alveolar bone (Lekovic, 2007).

Bone grafts divided into 4 major groups, autograft, allograft, xenograft, and synthetic graft (Torres *et al.*, 2011). Autografts and allografts are the best substitute for bone with defects, although there are several weaknesses of autograft and allograft (Randall, 2003). Many studies have been conducted to find alternatives to autograft and allografts, and overcome the weaknesses of it, one of which is by

using bovine xenograft which derived from bovine bones. It seems that is a good substitute due to the unlimited quantity, physical and chemical structure similar to human bones and low cost. The drawback is that the antigenic reaction that triggers excessive inflammation can arise in the use of these bovine bones (Zielak, 2002). One type of bovine xenograft that is used is freeze-dried bovine bone xenograft, in which the bones which are processed through a freezing and drying process with the aim of maintaining physical and chemical structures and to suppress the excessive antigenic reactions that can be caused.

The concept of bone healing after alveolar bone grafting is an inflammatory process in which

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inflammatory indicators involved like monocytes, lymphocytes, macrophages, and inflammatory mediators within blood circulation (Anderson, 2001). Within 1 to 3 days macrophages release cytokines Interleukin-1 (IL-1) and Tumor necrosis factor alpha (TNF- α). TNF- α cytokines produced in response to infection, antigen or injury and are one of the most abundant mediators in inflammatory tissue (Kokkas *et al.*, 2007). Research conducted by Paschalia and Antonios TNF- α concentration in experimental animals peaked 24 hours after bone damage and returned to normal levels after 72 hours (Paschalia, 2008). In addition, TNF- α is also produced as a result of grafting material that is considered a foreign object by the host. In excessive amounts, TNF- α molecules will stimulate bone resorption by inducing proliferation and differentiation of osteoclast progenitors and activating osteoclast formation indirectly. TNF- α also mediates tissue destruction by stimulating collagenase and degradation of type I collagen by fibroblasts. In connection with the aforementioned phenomenon, it is necessary to conduct research to find out whether there are differences in TNF- α secretion in human peripheral blood mononuclear cells (hPBMCs) after the freeze-dried bovine bone xenograft (FDBBX) administration on days 1,3 and 5.

MATERIALS AND METHODS

This is an *in vitro* study with Post Test Only Control Group research design, using human peripheral blood mononuclear cells (hPBMC) as samples divided into 2 groups, control group and experimental group. Control group: observation of TNF- α secretion in hPBMCs culture on days 1,3 and 5. Experimental group: observation of TNF- α secretion in FDBX 2.5% conditioned-medium application in hPBMCs culture media on days 1,3 and 5.

Preparation of Human PBMC

In this study hPBMCs culture was prepared from young healthy volunteers and has been screened for disease-free individual. Blood sample of 10 mL was aseptically taken from vena cubiti and collected in sterile EDTA containing tube. The blood was centrifuged 800 xg for 10 minutes at room temperature into which PBS was subsequently added at ratio 1:1. The blood was then put into conical tube containing Ficol-histopaque (Sigma

chemical co. Stl. Louis, MO) of 5 ML and centrifuged for 30 minutes. The PBMC layer formed was then collected and centrifuged again for 10 minutes. After cleansing with PBS twice, viability of hPBMC was determined with trypan blue exclusion (> 95%). hPBMC was then cultured in basis medium plus with cell density of 1×10^6 /mL for 24 hours

Preparation of FDBBX-conditioned medium

The FDBBX granules used in this study is produced by Tissue Bank, Dr. Soetomo Hospital, Surabaya, Indonesia with the granules size of 350 μ m and sterilized with gamma irradiation. Rehydration of FDBBX granules was done using alpha-MEM medium with concentration of 2.5% for 24 hours and centrifuged at 3,000 rpm for 10 minutes

Culture of hPBMC in FDBBX-conditioned medium

In treatment groups supernatant of the solution above was collected for filtration at porosities of 0.22 μ m. Samples of hPBMC was then cultured in microplate of 24 well (M-24) at cell density of 1×10^6 /mL in FDBBX-conditioned medium at the volume of 1 mL per well and incubated at 37° for 24 hours. Replication of 15 times was performed in treatment groups. In control group the hPBMC was cultured in basic medium with 15 replication incubated for 24 hours.

Measurement of TNF- α secretion

In this study, the amount of TNF- α secretion in sample cells was detected with human TNF- α monoclonal antibody (Biolegend, Legend MAXTM) and measured with ELISA reader by which in the first each sample was given a 100 μ L / well stopper solution. It was then transferred to be read on a microplate reader with a wavelength of 450 nm (in 30 minutes). Measurement using this tool will produce data in the form of optical density numbers. The optical density figure is the value of yellow sensitivity in the cell culture. The more intense the colour produced, the higher the optical density number. The yellow intensity that forms is directly proportional to the amount of TNF- α secretion.

The obtained data were analyzed using Kolmogorov-Smirnov and Levene test for normality and homogeneity, followed by t-test using SPSS software 24.0 (SPSS Inc, USA). Data results were expressed as means \pm SD. Values were considered statistically significant at $p < 0.05$ each group.

RESULTS

hPBMCs Culture

This study used culture preparations of human peripheral blood mononuclear cells (hPBMCs). Viability of hPBMCs was determined using trypan blue staining and obtained cell counts of 1×10^6 cell / mL (Figure 1).

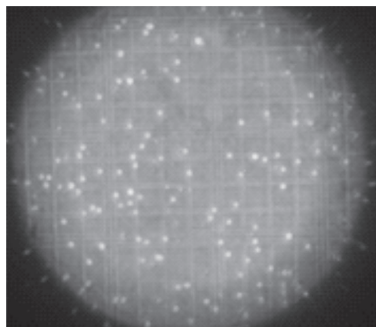


Fig. 1. hPBMCs in medium plus with a cell count of 1×10^6 cell/ mL (24 hours) seen using an inverted microscope

TNF- α secretion examination in hPBMCs culture

The results of ELISA reading it was shown that the mean value of TNF- α secretion in experimental (FDBBX-conditioned medium) group were all higher than the control group after 24 hours, 72 hours, and 120 hours of incubation. Statistical analysis with t-test revealed that there was significant difference in TNF- α secretion between the two groups in all observation periods with $p < 0.05$. It is noteworthy, however, that the TNF- α secretion are found to decrease with time in both experimental and control group (Figure 2).

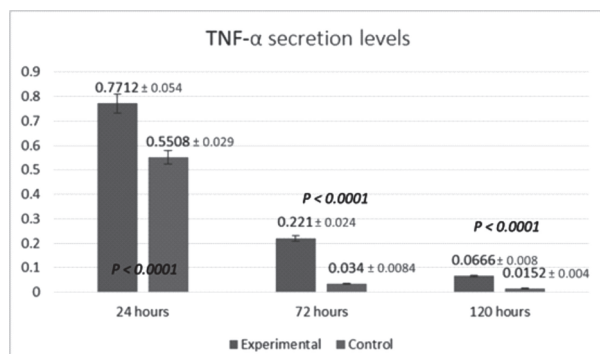


Fig. 2. The mean of TNF- α secretion levels and statistical comparison between the experimental and control group after 24 hours, 72 hours and 120 hours.
Note: * $p < 0.05$ = significant difference

DISCUSSION

Bone graft is a bone replacement material that is implanted in a bone defect through a surgical procedure to replace the lost bone with material from the patient's own body, another human body or natural substitutes for different species or synthetic materials. The use of bone graft is intended to form new bone (Nather, 2005). Freeze dried bovine bone xenograft (FDBBX) is a graft derived from bovine bones, processed by freezing and drying or also called lyophilization. Xenograft has a low apatite crystal structure ($100 \times 200 \text{ \AA}$) with 7% carbonate and a larger internal surface with a higher degree of pores making it more advantageous for the stability of the initial blood clots and increasing the migration of osteoblasts thereby increasing new bone formation that leads to osteogenesis (Scabbia, 2004). FDBBX used in this study because it has a high mineral and bone protein content. In bone proteins, there are BMPs growth factors that can induce Mesenchymal Stem Cells to proliferate and differentiate to form osteoblasts needed for new bone formation. In addition, the total xenograft porosity is 60% resembling human bone with a pore size of 300-1500 μm .

The freeze-dried bovine bone xenograft (FDBBX) studies *in vitro* requires the creation of a conditioned medium with optimal concentration so that solid powder or stem particles can be applied well to human Peripheral Blood Mononuclear Cells (hPBMCs) cultures. Based on preliminary research, freeze dried bovine bone- conditioned medium with 2.5% concentration gives the highest cell viability (unpublished data). Cell viability test is a comparison of the number of living cells and dead cells. Cell viability is often used as a cytotoxicity marker for a material (Freshney, 2000). Therefore, in this study FDBBX-conditioned medium was used with a concentration of 2.5%.

Tumor necrosis factor-alpha (TNF- α) is a cytokine produced by macrophages primarily in response to infection, antigen or injury TNF- α is a cytokine that is rapidly released after trauma or infection (ex. exposure to bacteria) and is one of the most abundant mediators in inflammatory tissue. Therefore, in this study TNF- α was used as an inflammatory mediator.

The concept of bone healing after alveolar bone grafting is an inflammatory process followed by bone healing in the form of proliferation and

differentiation of bone marrow mesenchymal stem cells which in turn will produce new bone tissue. The immune system and immune response play a very important role in the concept of bone healing. Peripheral Blood Mononuclear Cells (PBMCs) are blood cells that contain round nuclei in the form of lymphocytes, monocytes and macrophages. Peripheral Blood Mononuclear Cells (PBMCs) are important components in the immune system to fight infections and immune responses to foreign objects. Therefore, in this study human peripheral blood mononuclear cells (hPBMCs) were used as research subjects about the inflammatory response.

In this *in vitro* study, freeze dried bovine bone xenograft-conditioned medium was evaluated whether it caused a physiological or pathological inflammatory response by evaluating differences in TNF- α secretion levels and secretion patterns. Based on the results of the research that has been done, it was found that TNF- α secretion levels in hPBMCs after FDBBX conditioned medium on day 1 (24 hours), day 3 (72 hours), and day 5 (120 hours) is significantly different. The amount of TNF- α secretion increases on day 1 (24 hours), and decreases after day 3 (72 hours) and day 5 (120 hours). This is consistent with studies by Paschalia and Antonios, which showed TNF- α concentrations in experimental animals peaked 24 hours after bone damage and returned to normal levels after 72 hours. This is also in accordance with the theory that says in the process of bone healing, TNF- α expression and its receptors will follow a biphasic pattern. During this period, TNF- α is expressed by macrophages and other inflammatory cells. Increased TNF- α signals will induce secondary signals and produce chemotactic effects, activating cells that play an important role in bone regeneration processes (Paschalia, 2008)

In this study also, the obtained results show that there were significant differences between the control group and the experimental group at each treatment time. Increased TNF- α secretion in the experimental group compared to control group showed that TNF- α played a role in the inflammatory process in the healing phase after grafting. In addition, it can be caused by the presence ingredients of (FDBBX) which is considered a foreign object by the host. The presence of high protein content in (FDBBX) can also cause an immune reaction that can trigger excessive inflammatory reactions. In excessive amounts, TNF- α molecules will stimulate bone

resorption by inducing proliferation and differentiation of osteoclast progenitors and activating osteoclast formation indirectly; However, a decrease in TNF- α secretion on day 3 (after 72 hours) and day 5 (after 120 hours) compared to day 1 (24 hours) in this study could indicate that (FDBBX) a biocompatible bone graft material because the inflammatory response caused is physiological.

From the results of this study, it can also be seen in the control group consisting of non-stimulated human peripheral blood mononuclear cells (hPBMCs) that TNF- α secretion is still present. These results are consistent with a preliminary study which showed an increase in cytokine secretion in non-stimulated hPBMCs (Jansky, 2003). In this study it was also stated that TNF- α is the first cytokine secreted in non-stimulated hPBMCs which is likely to be secondary to the sampling procedure of hPBMCs and the procedure for making hPBMCs culture medium

CONCLUSION

This study demonstrates that Freeze-dried bovine bone xenograft conditioned medium at 2.5% concentration induces an increase in tumour Necrosis Factor- α secretion in human peripheral blood mononuclear cells, but does not cause prolonged inflammatory response.

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